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14. ABSTRACT In this annual progress report of the work performed from 7/15/2014 to 7/14/2015, we: i) present data on a unique subset of ovarian cancers which are not BRCAlike, i.e. not HR deficient and are resistant to PARPi but are sensitive to platinum. These tumors exhibit alterations in another DNA repair pathway called nucleotide excision repair (NER) pathway. ii) present data that HSP90-inhibitors which were identified via our BRCAness profile may sensitize PARPi-resistant xenograft models to PARPi. iii) update our progress on validating our profile in a cohort of molecularly defined BRCA-mutated and HR-proficient tumors.				
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1. INTRODUCTION

Patients with BRCA1/2-associated EOCs exhibit improved overall survival and high sensitivity to double strand DNA break inducing agents due to an underlying defect in DNA repair via HR. However, it is increasingly recognized that a subset of patients with sporadic EOCs also exhibit defective HR caused by mechanisms that are unrelated to germline BRCA1 or BRCA2 mutations. These tumors may behave similarly to BRCA1/2-mutated EOCs and are commonly referred to as having a "BRCAness" phenotype (i.e. BRCAlike). Identifying tumors with a BRCAness phenotype is of increased clinical importance not only due to the advent of PARP-inhibitors but also because patients with this phenotype may need to be managed differently than the remaining patients. We have developed a 60-gene expression profile that may identify tumors with a BRCAness phenotype and may also be used to identify compounds that can enhance responsiveness to platinum and PARP-inhibitors. In this annual progress report of the work performed from 7/15/2014 to 7/14/2015, we:

- i) present data on a unique subset of ovarian cancers which are not BRCAlike, i.e. not HR deficient and thus are resistant to PARPis but are sensitive to platinum.
- ii) present data that HSP90-inhibitors which were identified via our BRCAness profile may sensitize PARPi resistant xenograft models to PARPis.
- iii) update our progress on validating our profile in a cohort of molecularly defined BRCA-mutated and HR-proficient tumors.

2. KEYWORDS

Ovarian cancer, Homologous recombination, BRCAness, Gene expression profiling, PARP inhibitors, Platinum analogues, HSP90 inhibitors, Platinum Resistance, Nucleotide Excision Repair

3. ACCOMPLISHMENTS

- **What were the major goals of the project?**

The major goals of this project during this period were to continue our work along Tasks 1, 3 and 4. The first goal was to identify subsets of ovarian cancers which are BRCAlike or nonBRCAlike and to further characterize their sensitivity to platinum and PARPi (Task 1). Another goal was to provide further data showing that HSP90-inhibitors sensitize ovarian cancers to PARPi (Task 3). Finally, in our Task 4, our goal was to continue working towards validating our profile in a cohort of molecularly defined BRCA-mutated and HR-proficient tumors.

- **What was accomplished under these goals?**

We have fully accomplished the goals of this project during this period.

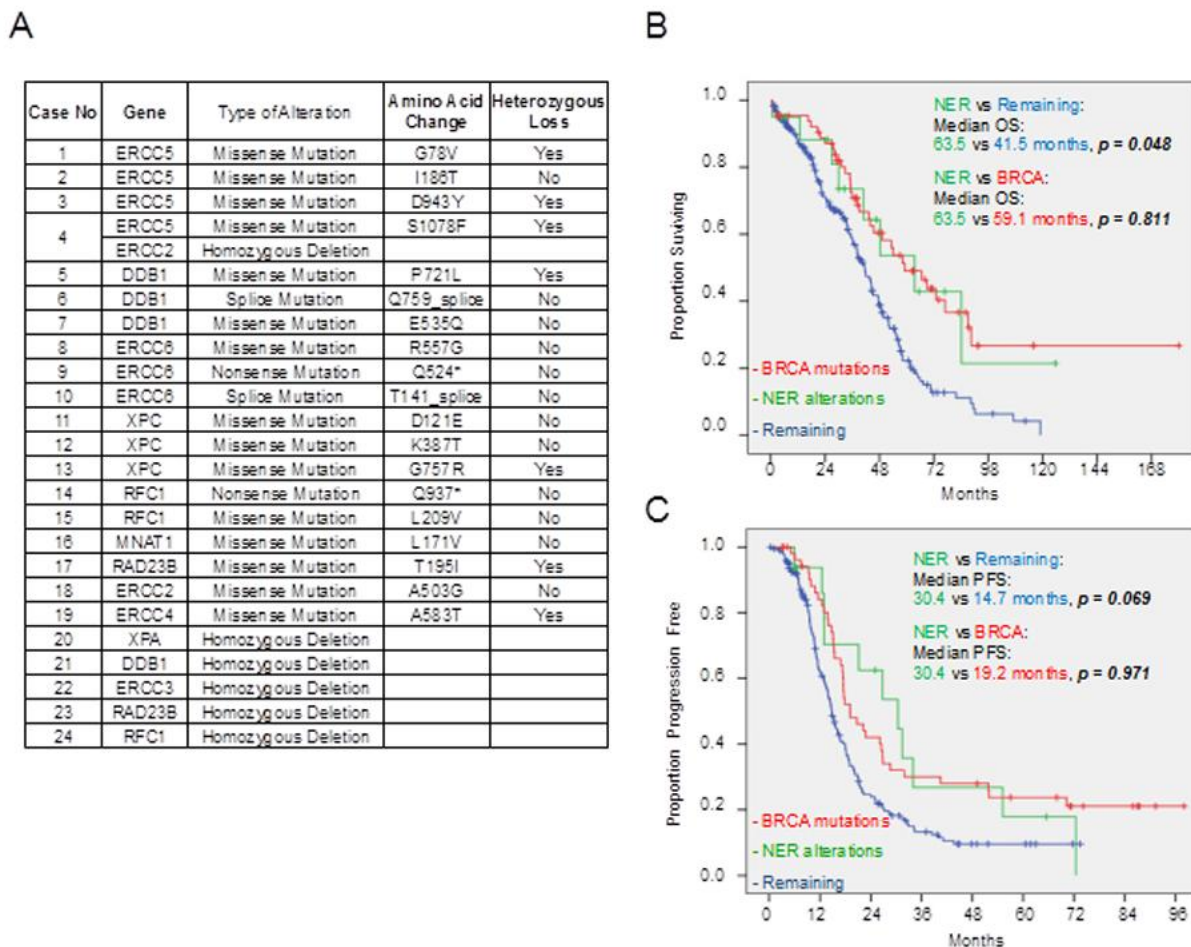
Task 1. Determine whether the BRCAness gene expression profile is capable of prospectively identifying sporadic patients whose tumors exhibit defects in homologous recombination and increased sensitivity to platinum and PARP inhibitors in vitro.

In addition and in continuation of the work we have performed the previous years, we were able to define a unique subset of ovarian cancers which are not BRCAlike, i.e. not HR deficient and thus are resistant to PARPi but are sensitive to platinum. Specifically, we curated the EOC TCGA dataset to assess potential inactivating events of the nucleotide excision repair (NER) pathway including mutations, homozygous deletions and promoter hypermethylation of NER genes. We found that a total of 24 (8%) of 316 EOCs harbored either NER mutations or homozygous deletions of NER genes (Fig. 1A). Specifically, we identified 19 cases with nonsynonymous or splice site NER gene mutations (all somatic) and 6 cases with homozygous deletions of NER genes among the 316 sequenced EOCs of the TCGA dataset. None of the NER genes were found to harbor promoter hypermethylation. All NER mutations were mutually exclusive, i.e. no individual tumor harbored mutations in more than one NER gene. Furthermore, NER mutations were mutually exclusive with homozygous deletions of the NER genes with the exception of one case that harbored both an ERCC5 mutation and homozygous deletion of ERCC2. Of the 19 cases with NER mutations, 7 (36.8%) were accompanied by heterozygous loss of the respective NER gene, indicating that in these cases both wild-type alleles had been lost. Importantly, patients with tumors with NER alterations exhibited higher median OS (63.5 vs

41.5 months respectively, log rank $p = 0.048$) and PFS (30.4 vs 14.7 months respectively, log rank $p = 0.069$) compared to patients with tumors without NER alterations and BRCA1/2 mutations (Fig. 1B and 1C). Furthermore, patients with tumors with NER alterations exhibited similar outcome (OS and PFS) with tumors harboring BRCA1 or BRCA2 mutations (Fig. 1B and 1C).

As a proof of principle that NER alterations are functionally associated with platinum sensitivity, we evaluated one NER mutation (ERCC6-Q524*) that was present in a patient with stage IV suboptimally-debulked high grade serous tumor that had complete response to first line platinum chemotherapy and remained in complete remission for 31.5 months after diagnosis.

Figure 1.

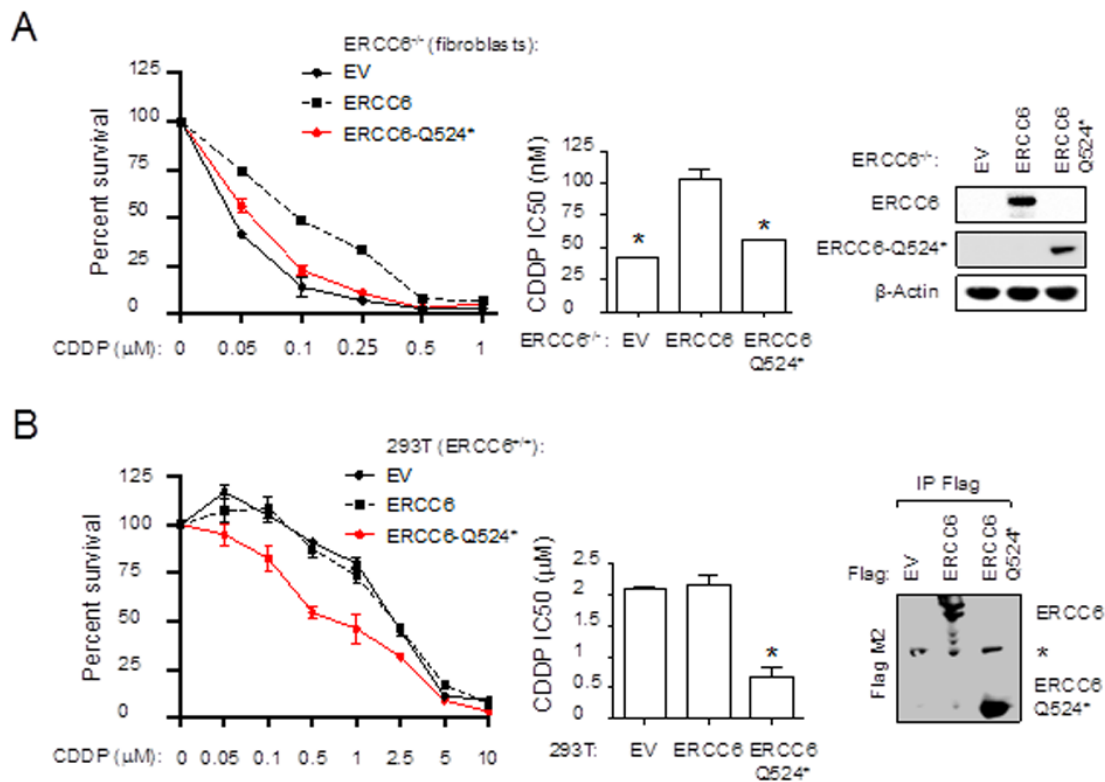


In order to determine the functional significance of ERCC6-Q524* on platinum sensitivity, we evaluated whether this variant could rescue platinum sensitivity in an ERCC6-deficient cell line. An ERCC6-deficient immortalized fibroblast cell line (GM16095) was complemented with either

wild-type ERCC6 or the mutant ERCC6-Q524*. Expression of wild-type ERCC6 rescued cisplatin sensitivity of ERCC6-deficient cells while complementation with mutant ERCC6-Q524* did not impact cisplatin sensitivity (Fig. 2A). In order to confirm that ERCC6 loss alone is solely sufficient to induce cisplatin sensitivity, we assessed cisplatin cytotoxicity following siRNA knockdown of ERCC6. ERCC6 depletion significantly increased platinum sensitivity, comparable to BRCA2 loss, a major mediator of DNA crosslink repair.

Given that ERCC6-Q524* was a somatic mutation and not associated with heterozygous loss, we evaluated whether this mutation may exert a dominant negative effect. We postulated that ERCC6-Q524* may interfere with the function of the wild-type allele and hence increase sensitivity to cisplatin. Indeed, introduction of the ERCC6-Q524* variant in ERCC6 wild-type 293T cells dramatically increased cisplatin sensitivity compared to cells transfected either with wild-type ERCC6 or control empty vector suggesting that this mutation sensitizes cells to cisplatin by a dominant negative mechanism (Fig. 2B).

Figure 2.



We evaluated the association of this NER mutation with sensitivity to the PARPi rucaparib. Unlike in the case of cisplatin, expression of wild-type or mutant ERCC6 did not affect PARPi

sensitivity of ERCC6-deficient fibroblasts. Furthermore, since defective HR is a critical mediator of platinum and PARPi sensitivity in EOC, we evaluated whether deficiency in ERCC6 affected HR in vitro. Inhibition of ERCC6 did not affect HR efficiency in vitro, as measured by direct-repeat GFP recombination (DR-GFP) assay and by IR-induced RAD51 foci formation, a surrogate for HR efficiency. Together, these results indicate that functional loss of ERCC6 does not impair HR efficiency nor alters sensitivity to PARPi or other double strand break-inducing agents such as doxorubicin.

In conclusion, we report for the first time that NER pathway alterations (mutations and homozygous deletions) occur in EOC and that these alterations are associated with a phenotype of clinical platinum sensitivity that is similar to that of BRCA1/2-mutated tumors characterized by improved overall and progression free survival. We showed that the NER mutation identified in a very platinum sensitive tumor (ERCC6-Q524*) was functionally associated with platinum sensitivity in vitro. Importantly, this mutation did not affect HR and did not confer sensitivity to PARPi thus providing a novel mechanism of discordance between platinum and PARPi sensitivity in EOC. Our findings suggest that NER alterations may have a previously unrecognized role as biomarkers for selection of patients for participation in PARPi trials as well as for deciding therapy after development of PARPi resistance. This work was published at Cancer Research (Ceccaldi R, O'Connor KW, Mouw KW, Li AY, Matulonis UA, D'Andrea AD, Konstantinopoulos PA. Cancer Res. 2015 Feb 15;75(4):628-34).

Task 2. Determine whether the BRCAness gene expression profile is associated with clinical response to platinum and survival in patients with sporadic ovarian cancer.

This task has already been completed.

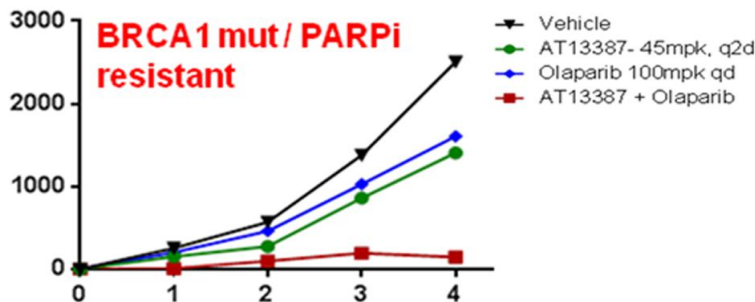
Task 3. Evaluate whether the compounds identified by the Connectivity Map can reverse PARP resistance in vitro, and to investigate the mechanism for this effect.

In addition to the in vitro data, we have now performed experiments to assess whether HSP90 inhibitors identified via the Connectivity Map can revert PARPi resistance in vivo.

Specifically, we used a PARPi resistant xenograft model and we performed efficacy studies. Strikingly, this model was PARPi resistant and was also resistant to combinations of olaparib and the PI3K inhibitors BKM120 or BYL719.

Olaparib was dosed at 100mg/kg po daily x 4 weeks, AT13387 (HSP90i) was administered at 45mg/kg po for 2 days (D1,D2) on / 5 days off x 4 weeks (i.e. Days 1, 2, 8, 9, 15, 16, 22, 23). As shown in Figure 3, the combination of AT13387 and olaparib induced inhibition of tumor growth as opposed to vehicle control, olaparib alone and AT13387 alone.

Figure 3.



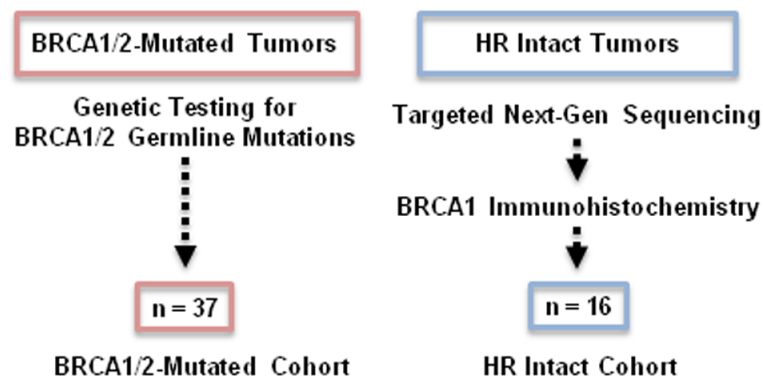
Task 4. Determine the reproducibility of the BRCAness profile when using the DASL mRNA assay in a cohort of FFPE ovarian cancer specimens with known clinical outcome and platinum responsiveness.

A very important step towards assessing the reproducibility of our BRCAness profile is to identify the right cohort of molecularly defined patients that are BRCAlike and nonBRCAlike. To achieve this, we defined two groups of patients, a BRCA1/2-mutated group which is expected to be BRCAlike and a HR-proficient group which is expected to be nonBRCAlike.

The BRCA1/2-mutated group was comprised of 37 HGSOCs (29 with BRCA1 and 8 with BRCA2 mutations) with BRCA1/2 germline mutations identified by genetic testing (Figure 2). The HR-proficient group (i.e. group of tumors without HR alterations) comprised 16 ovarian cancers which were identified in a two-step process (Figure 2). First, we performed Next Generation Sequencing (NGS) to exclude tumors with mutations in HR genes; this analysis identified 17 such tumors (Figure 4). These 17 tumors were subsequently evaluated for BRCA1 expression by immunohistochemistry to exclude the possibility of BRCA1 promoter hypermethylation that would lead to absent BRCA1 expression. As a result of this testing, 1 tumor was found to have staining in less than 5% of tumor cells with the presence of a strong internal control (Figure 3), which was then excluded from the HR-proficient(HR intact) group. Interestingly, review of the

NGS data for this case demonstrated that this tumor had a single copy deletion of the BRCA1 gene, suggesting that BRCA1 loss was likely due to single copy deletion of BRCA1 and epigenetic silencing of the complementary allele. Ultimately, the HR proficient group consisted of 16 tumors without mutations in HR pathway genes and without BRCA1 loss by IHC.

Figure 4.



Since we have now identified the right molecularly-defined cohort of patients, we plan to determine the reproducibility of our BRCAness profile.

○ **What opportunities for training and professional development has the project provided?**

Nothing to report.

○ **How were the results disseminated to communities of interest?**

Nothing to report.

○ **What do you plan to do during the next reporting period to accomplish the goals?**

In the next funding period, we plan to complete Tasks 1, 3 and 4.

4. IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**

a) We have discovered a novel mechanism of discordance between platinum and PARPi sensitivity in ovarian cancer that involved NER alteration. Our findings suggest that NER alterations may have a previously unrecognized role as biomarkers for selection of patients for participation in PARPi trials as well as for deciding therapy after development of PARPi resistance.

b) The promise of PARP inhibitors in the management of ovarian cancer is tempered by the fact that HR-proficient cancers do not respond well to these agents, suggesting that approximately 50% of ovarian cancer patients (i.e. those without HR alterations) do not benefit from this novel class of drugs. Combination of PARPis with agents that inhibit HR may represent an effective strategy to sensitize HR proficient tumors to PARPis and thus potentially expand use of these agents beyond patients with HR deficient EOCs. Our in vivo findings that HSP90is revert PARPi resistance in xenografts provide the preclinical rationale for using a combination of 17-AAG and olaparib and/ or carboplatin in ovarian cancers that are HR proficient either at baseline or at the time of development of platinum or PARPi resistance. This can have a significant impact on patients who develop resistance to PARP-inhibitors or platinum analogues as the combination of 17-AAG/PARP-inhibitors or 17-AAG/carboplatin may effectively overcome this problem.

- **What was the impact on other disciplines?**

Nothing to report.

- **What was the impact on technology transfer?**

Nothing to report.

- **What was the impact on society beyond science and technology?**

Nothing to report.

5. CHANGES/PROBLEMS

- **Changes in approach and reasons for change**

Nothing to report.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

Nothing to report.

- **Changes that had a significant impact on expenditures**

Nothing to report.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to report.

- **Significant changes in use or care of human subjects**

Nothing to report.

- **Significant changes in use or care of vertebrate animals.**

Nothing to report.

- **Significant changes in use of biohazards and/or select agents**

Nothing to report.

6. PRODUCTS

- **Publications, conference papers, and presentations**

Journal publications.

1. Ceccaldi R, O'Connor KW, Mouw KW, Li AY, Matulonis UA, D'Andrea AD, Konstantinopoulos PA. A Unique Subset of Epithelial Ovarian Cancers with Platinum Sensitivity and PARP Inhibitor Resistance. Cancer Res. 2015 Feb 15;75(4):628-34.

2. Ceccaldi R, Liu JC, Amunugama R, Hajdu I, Primack B, Petalcorin MI, O'Connor KW, Konstantinopoulos PA, Elledge SJ, Boulton SJ, Yusufzai T, D'Andrea AD. Homologous-

recombination-deficient tumours are dependent on Polθ-mediated repair. Nature. 2015 Feb 12;518(7538):258-62. doi: 10.1038/nature14184

3. Guillemette S, Serra RW, Peng M, Hayes JA, Konstantinopoulos PA, Green MR, Cantor SB. Resistance to therapy in BRCA2 mutant cells due to loss of the nucleosome remodeling factor CHD4. Genes Dev. 2015 Mar 1;29(5):489-94.

4. Howitt BE, Shukla SA, Sholl LM, Ritterhouse LL, Watkins JC, Rodig S, Stover E, Strickland KC, D'Andrea AD, Wu CJ, Matulonis UA, Konstantinopoulos PA. Association of Polymerase ϵ -Mutated and Microsatellite-Unstable Endometrial Cancers With Neoantigen Load, Number of Tumor-Infiltrating Lymphocytes, and Expression of PD-1 and PD-L1. JAMA Oncol. 2015 Jul 9. doi: 10.1001/jamaoncol.2015.2151

5. Konstantinopoulos PA, Ceccaldi R, Shapiro GI, D'Andrea AD. Homologous Recombination Deficiency: Exploiting the Fundamental Vulnerability of Ovarian Cancer. Cancer Discov. 2015 Oct 13

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers, and presentations.

1. Kyle Strickland, Brooke E. Howitt, Scott J. Rodig, Lauren Ritterhouse, Alan D. D'Andrea, Ursula Matulonis, Panagiotis Konstantinopoulos. Tumor infiltrating and peritumoral T cells and expression of PD-L1 in BRCA1/2-mutated high grade serous ovarian cancers. ASCO 2015 Meeting, Oral Presentation

Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

Nothing to report.

Inventions, patent applications, and/or licenses

Nothing to report.

Other Products

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Panagiotis Konstantinopoulos: No change

As a reminder, given that Dr Konstantinopoulos moved to Dana Farber Cancer Institute, this award was transferred to Dana Farber Cancer Institute effective 9/1/2014.

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report.

- **What other organizations were involved as partners?**

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report.